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New high affinity peptide antagonists to the spinal galanin receptor

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1 The role of endogenous galanin in somatosensory processing has been studied with galanin receptor antagonists. The new galanin receptor ligands C7, M32, M38 and M40 bind with high affinity (K_d in nanomolar range) to spinal cord galanin receptors and possess oxidative stability as compared to earlier generations of peptide ligands. These peptides have been examined in the spinal flexor reflex model where exogenous galanin exhibited biphasic excitatory and inhibitory effects.

2 Intrathecal administration of C7 [galanin(1–13)-spantide] and M32 [galanin (1–13)-neuropeptide Y(25–36) amide] blocked facilitation of the nociceptive flexor reflex induced by 30 pmol intrathecal galanin in decerebrate, spinalized rats in a dose-dependent manner, thus behaving as antagonists of the galanin receptor. In contrast, M38 [galanin(1–13)-(Ala-Leu)$_3$-Ala amide] and M40 [galanin(1–13)-Pro-Pro-(Ala-Leu)$_2$-Ala amide], exhibited only weak antagonism at high doses in this model. Moreover, lower doses of M40 potentiated galanin-induced reflex facilitation. C7 was neurotoxic at high doses in the rat spinal cord.

3 M32 and C7 were potent antagonists of galanin receptors in rat spinal cord, in correlation with their *in vitro* binding characteristics. In contrast, M38 and M40, despite their high *in vitro* affinity, exhibited only very weak antagonism. Moreover, M40 may also behave as a partial agonist.

4 Previous studies have shown that the galanin receptor may be heterogeneous. The discrepancy between *in vitro* binding and *in vivo* antagonistic potency of M38 and M40 may also suggest the presence of different galanin receptor subtypes within the rat spinal cord. However, other explanations for the discrepancy, such as differences in metabolic stability, diffusion rates and penetration to the site of action are also possible.

Keywords: Flexor reflex; galanin; galanin receptor; antagonist; pain; spinal cord

Introduction

Since its discovery in 1983 (Tatemoto *et al.*, 1983), galanin has received much attention as a potent neuropeptide with widespread distribution in the endocrine, peripheral and central nervous systems (Ch'ng *et al.*, 1985; Melander *et al.*, 1986; Rossowski *et al.*, 1990; Bartfai *et al.*, 1992; 1993a). Numerous properties and functions of galanin have been identified by use of exogenously applied synthetic peptides (Bartfai *et al.*, 1992; 1993a). In the somatosensory system, galanin occurs in a relatively small population of dorsal root ganglion cells, primarily with small somata (Ch'ng *et al.*, 1985; Skofitsch & Jacobowitz, 1985). Previous studies have indicated that galanin has a complex, biphasic effect upon nociceptive transmission at spinal level (Wiesenfeld-Hallin *et al.*, 1992a).

The synthesis of the first generation of galanin receptor antagonists was based on the finding that the biological activity of the full length peptide, galanin(1–29), resides in the N-terminus (Fisone *et al.*, 1989; Crawley *et al.*, 1990; Xu *et al.*, 1990) and that its N-terminal, as well as C-terminal, parts from two α-helices separated by Pro[13] (Rigler *et al.*, 1991). These compounds were chimeric, bioreceptor recognizing peptides with galanin(1–13) as the N-terminal fragment and the carboxy-terminus of some other bioactive peptides whose activity was known to reside in the C-terminus (Bartfai *et al.*, 1991; 1992; 1993b; Langel *et al.*, 1992). Two of these chimeric peptides, M15 [galanin(1–13)-substance P(5–11) amide] and M35 [galanin(1–13)-bradykinin(2–9) amide], have been extensively tested in the spinal flexor reflex model (Bartfai *et al.*, 1991; Wiesenfeld-Hallin *et al.*, 1992b) and both peptides dose-dependently antagonized intrathecal (i.t.) galanin-induced facilitation of the flexor reflex. Moreover, M15 and M35 were also able to reverse galanin-induced antagonism of reflex hyperexcitability following conditioning stimulation (CS) of unmyelinated afferents. M35, by blocking the effect of endogenous galanin, potentiated C-fibre CS-induced flexor reflex facilitation (Wiesenfeld-Hallin *et al.*, 1992b). Both M15 and M35 have been shown to be high-affinity antagonists (K_D = 0.1 nM) at the spinal galanin receptor (Bartfai *et al.*, 1991; Wiesenfeld-Hallin *et al.*, 1992b), with Hill coefficients of unity, indicating a single class of recognition sites.

To evaluate further the structural requirements for high-affinity binding and functional agonism/antagonism we have studied the displacement of [^{125}I]-galanin from spinal galanin receptors and the effects on the spinal flexor reflex of four additional chimeric peptides/galanin analogues. The peptides studied here were: M32, consisting of galanin(1–13)-NPY(25–36) amide; C7, galanin(1–13)-spantide; M40, galanin(1–13)-Pro-Pro-(Ala-Leu)$_2$-Ala amide and M38, galanin(1–13)-(Ala-Leu)$_3$-Ala amide. The peptides with non-sense carboxy terminals, M38 and M40, have been designed in order to avoid possible bi-receptor recognition and activation, as well as to stabilize the interaction of the ligand with the galanin receptor.

Methods

Protocol of the electrophysiological study

Twenty female Sprague-Dawley rats (200–250 g, ALAB, Sweden) were used. The animals were initially briefly anaesthetized with methohexital (Brietal, Lilly, Indianapolis, USA, 70 mg kg^{-1}, i.p.), ventilated and decerebrated by aspiration of the forebrain and midbrain. The spinal cord was exposed by a laminectomy at mid-thoracic level and sectioned at Th8-9. An i.t. catheter (PE 10) was implanted caudally to the transection with its tip on the lumbar spinal cord (L4–5). The flexor reflex was elicited by supramaximal electric shocks to the sural nerve or its innervation area in the left foot (0.5 ms, 10 mA, 1 min^{-1}) that activated A- and C-afferents. In some experiments, a train of CS (0.9 Hz, 20 stimuli) of the same strength as the test stimulus was administered to facilitate briefly the magnitude of the reflex (Wall & Woolf, 1984).

The flexor reflex was recorded as EMG activity via stainless steel needle electrodes inserted into the ipsilateral posterior biceps femoris/semitendinosus muscles. The number of action potentials elicited during the reflex was integrated over 2 s and recorded on a chart recorder. During the experiments the heart rate and rectal temperature of the rat were monitored.

Synthetic peptides

The structure of the chimeric peptides used in the present study is summarized in Table 1. The peptides were assembled in a stepwise manner on a solid support using an Applied Biosystems Model 431A Peptide Synthesizer and the standard DCC/HOBt Solvent-Activation strategy on a 0.1 mmol scale (small scale). *tert*-Boc-amino acids were coupled to MBHA (Bachem Feinchemikalien AG, Switzerland) resin as hydroxybenzotriazole (HOBt) esters.

Deprotection, cleavage and purification of the peptides has been described earlier (Langel *et al.*, 1992). Purity of the individual peptides was checked by analytical Nucleosil 120-3 C_{18} reversed-phase h.p.l.c. column (0.4 cm × 10.0 cm) and determined to be ≥98%. Molecular weights of the peptides were determined with Plasma Desorption Mass Spectrometer (PDMS) Model Bioion 20, Applied Biosystems, the calculated values were obtained in each case.

Binding studies

Membrane preparation from rat spinal cord and receptor binding analysis was carried out by filtration technique as described earlier (Land *et al.*, 1991), using 5 mM HEPES-buffered (pH 7.4) Krebs-Ringer solution containing 1 mg ml^{-1} bacitracin and 0.5 mg ml^{-1} bovine serum albumin (BSA). The mixtures were incubated for 30 min at 37°C with 0.1–0.2 nM porcine [^{125}I]-galanin (NEN, specific activity 2200 Ci mmol^{-1}) as a tracer at increasing concentrations (0.001–1000 nM) of the peptides to be tested. Specific binding was defined as that displaceable by 1 mM galanin. The K_d values of the displacing ligands were calculated from the computer-generated IC_{50} values using the correction of Cheng & Prusoff (1973). Fitting of the experimental data was carried out be means of a non-linear least squares method using the programme Kaleidograph on a Macintosh SE/30.

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Results

Data presented in Table 1 and Figure 1 demonstrate that all four peptides/galanin analogues tested, C7, M32, M38 and M40, are high-affinity ligands at the spinal cord galanin receptors characterized with K_d values ranging from 0.01–6.8 nM. The displacement of [^{125}I]-galanin by some of the ligands, C7, M32 and M38, was characterized with Hill coefficients of 0.3–0.5, being significantly below unity (*vs* n_H = 1 for galanin and M40), indicating that the galanin recognition sites in spinal cord can be heterogeneous.

In order to evaluate the functional nature (antagonists *vs* agonists) of these galanin receptor ligands, we have determined the effect of these ligands on the galanin induced (30 pmol, i.t.) facilitation of the flexor reflex. It has been demonstrated earlier (Wiesenfeld-Hallin *et al.*, 1989) that i.t. galanin exerts a complex dose-dependent effect on the spinal flexor reflex; facilitatory at low doses (30–300 pmol) and inhibitory at high doses (30 nmol).

I.t. galanin (30 pmol) briefly facilitated the flexor reflex (Figure 2), with an average peak reflex facilitation of 115.2 ± 15.4% above baseline, lasting 5.7 ± 0.5 min (n = 19). Both M32 and C7 applied i.t. dose-dependently blocked reflex facilitation induced by i.t. galanin (Figure 3) with ED_{50} of 71.5 pmol for M32 and 23.2 pmol for C7. M38 only weakly antagonized galanin-induced reflex facilitation, with a ≈100 fold lower inhibitory potential (ID_{50} = 2.1 nmol) than M32 and C7 (Figure 3). In contrast, M40 potentiated galanin-induced

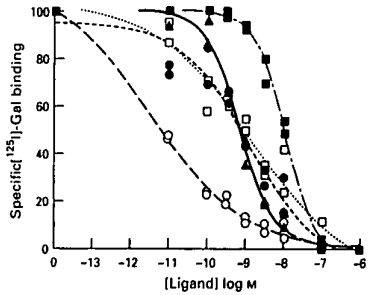

Figure 1 The inhibition of specific [^{125}I]-galanin (0.1–0.2 nM) binding to membranes from lumbar dorsal spinal cord by galanin (▲), M32 (○), M38 (□), M40 (■) and C7 (●), at 37°C for 30 min, with increasing concentrations of displacing ligand under equilibrium conditions. Non-specific binding was defined as that displaceable by 1 μM galanin. Two independent experiments were performed and each value was dertermined in duplicates.

Table 1 Displacement of 0.1–0.2 nM [^{125}I]-galanin from rat spinal cord membranes by galanin receptor ligands

Peptide	*Sequence*	K_d, (nM)	n_H
Rat galanin	GWTLNSAGYLLGP-HAIDNHRSFSDKHGLT amide	0.53 ± 0.2	0.97 ± 0.03
M32 Galanin(1-13)-NPY(26-36)	GWTLNSAGYLLGP-RHYINLITRQRY amide	0.010 ± 0.001	0.33 ± 0.06
M38 Galanin(1-13)-ALALALA	GWTLNSAGYLLGP-ALALALA amide	0.70 ± 0.07	0.41 ± 0.12
M40 Galanin(1-13)-PPALALA	GWTLNSAGYLLGP PPALALA amide	6.8 ± 2.2	0.93 ± 0.13
C7 Galanin(1-13)-Spantide	GWTLNSAGYLLGP-(D-R)PKPQQ(D-W)F(D-W)LL amide	1.16 ± 0.12	0.46 ± 0.04

The data are the means ± s.e.mean from two independent experiments and each value was determined in duplicate. Specific binding was defined as that displaceable by 1 μM unlabelled galanin, representing 80–90% of total binding.

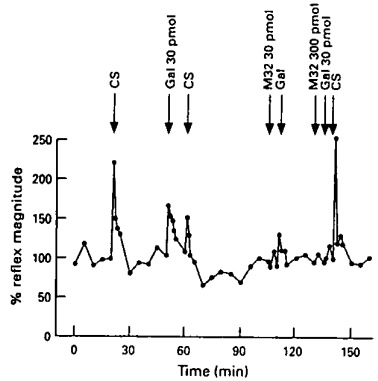

Figure 2 Illustration of the effect of conditioning stimulation (CS) of unmyelinated afferents, GAL and M32 in rat spinal cord. The baseline flexor reflex was defined as 100%. Note that i.t. GAL by itself facilitated of the flexor reflex, but it also antagonized the reflex facilitation by the C-fibre CS. Both actions of GAL were blocked by M32.

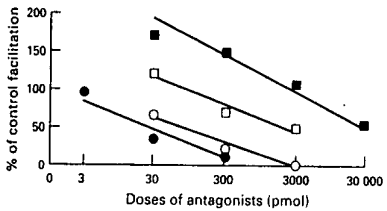

Figure 3 Summary of the effects of C7 (●), M32 (○), M38 (□) and M40 (■) on the facilitation of the flexor reflex induced by 30 pmol i.t. GAL. Data from 3–4 experiments are presented for each dose and expressed as mean ± s.e.m. The regressions for all compounds were calculated. C7; $y = -35.8x + 98.8$ ($P < 0.01$), M32; $y = -32.2x + 109.7$ ($P < 0.001$), M38; $y = -35.8x + 169.2$ ($P < 0.05$) and M40; $y = -48.2x + 269.5$ ($P < 0.01$). The ED_{50} values with 95% confidence limits are 23.2 pmol (6.7–42.2 pmol) for C7; 71.5 pmol (22.1–178.4 pmol) for M32; 2.1 nmol (178.4 pmol – ∞) for M38 and 39.8 nmol (7.9 nmol – ∞) for M40.

facilitation at low doses, but attenuated it at very high doses, thus behaving as a mixed agonist-antagonist at the spinal galanin receptor(s).

Besides the galanin-induced complex facilitatory-inhibitory effect on the flexor reflex, i.t. galanin dose-dependently (30 pmol–30 nmol) depressed the reflex facilitation induced by the C-fibre CS (Wiesenfeld-Hallin *et al.*, 1989). I.t. galanin (30 pmol) applied prior to the CS caused a brief reflex facilitation and significantly inhibited the subsequent CS-induced facilitation (Figure 2). This galanin receptor-mediated depression was also potently reversed by M32 and C7, confirming their antagonistic properties (Figure 2). Furthermore, C7 at a high dose (3 nmol) totally blocked the flexor reflex, such that the reflex response to strong, even tissue damaging, stimuli could not be evoked for up to 60 min after drug administration, indicating the presence of neurotoxicity. All the chimeric ligands caused brief facilitation of the flexor reflex upon i.t. injection. However, this facilitation could be correlated with neither affinity nor antagonism.

Discussion

In earlier studies we have shown the presence of a G-protein coupled single class of galanin receptors in the spinal cord, with a K_d for galanin of 0.6 nM present in a concentration of ≈50 fmol mg^{-1} protein (Bedecs *et al.*, 1992). In the present study we have evaluated the binding properties and functional effects of a series of galanin chimeras which in their C-terminal contain either other bioactive peptides (C7 and M32) or non-sense sequences (M38 and M40). All the chimeric peptides displaced [^{125}I]-galanin from rat spinal cord membranes with high affinities, in the range of K_d from 0.01 to 6.8 nM (Table 1).

M32, M38 and C7 had K_d values of 0.01 nM, 0.7 nM and 1.16 nM with Hill coefficients of 0.33, 0.41 and 0.46, respectively, showing a clear correlation between high-affinity recognition and low Hill coefficients, indicating heterogeneity of recognition sites in the spinal cord. M32 actually showed the highest affinity toward a galanin receptor yet reported in any system. However, in the electrophysiological studies, only M32 and C7 potently antagonized the galanin-induced facilitation of the flexor reflex, whereas M38 and M40 only weakly or partly antagonized the effect of galanin. The antagonistic potencies of M32 and C7 are comparable with those of M15 and M35, two previously tested chimeric galanin receptor antagonists (Bartfai *et al.*, 1991; Wiesenfeld-Hallin *et al.*, 1992b), although significantly differing from these in binding properties since M15 and M35 both displaced [^{125}I]-galanin in spinal cord membranes with Hill coefficients of unity.

M32 and C7 also potently reversed the galanin-mediated depression on C-fibre CS-induced reflex facilitation, their effective doses being comparable with M15 and M35. This suggests that spinal cord galanin receptors mediating the excitatory and inhibitory effects on the flexor reflex are similar in their structural requirement for functional agonism and antagonism and cannot be distinguished by the structurally divergent chimeric antagonists used so far.

An interesting finding is that both C7 [galanin(1–13)-spantide] and M15 [galanin(1–13)-substance P(5–11 amide)] (Bartfai *et al.*, 1991) behaved as galanin receptor antagonists in the spinal cord, thus excluding any substance P receptor interaction, since the galanin receptor antagonism is similar if the C-terminal part of the galanin chimera contains a substance P agonist [substance P(5–11)] or an antagonist (spantide). However, C7, which in its C-terminal contains the entire spantide molecule, was shown to be neurotoxic at high doses. Previous studies in this and other laboratories have shown that i.t. neuropeptides and neuropeptide antagonists could be toxic at high concentrations, in particular the substance P receptor antagonist, spantide (Post & Paulsson, 1985; Wiesenfeld-Hallin & Duranti, 1987). Thus, the neurotoxicity of C7 is most probably due to spantide on its C-terminal.

In contrast to the efficacy of M32 and C7, the other two chimeras, M38 and M40, exhibited low or no antagonism, respectively. M38, though having an affinity of 0.7 nM at spinal cord galanin receptors, could block the galanin-induced facilitation only slightly even at quite a high dose (30 nmol), thus behaving as a weak antagonist. The potentiation of galanin-induced facilitation by M40 at low doses and the fact that a thousand fold excess of M40 was required to produce moderate antagonism of galanin-mediated reflex facilitation indicate that M40 is probably a partial agonist with a weak antagonistic property in this system.

The fact that only the chimeras containing bioactive peptides in their C-terminal, M32 [NPY (25–36)] and C7 (spantide), behaved as antagonists favours a bi-receptor interaction-mediated galanin receptor antagonism (Langel *et al.*, 1992). This is excluded for C7 (see above). However, in the case of M32, this is not clear as the Hill coefficient in the [^{125}I]-galanin displacement experiment was significantly below unity, indicating putative multiple recognition sites of M32. Autoradiographic and functional studies have shown the presence of NPY receptors on DRG cells and in the dorsal horn of the spinal cord (Kar & Quirion, 1992), where NPY was shown to reduce depolarization-induced SP-release through an inhibition of Ca^{2+} currents (Walker *et al.*, 1988). I.t. NPY has antinociceptive effects and depresses the spinal flexor reflex (Hua *et al.*, 1991; Xu *et al.*, 1994). These studies have suggested a possible presynaptic localization of NPY binding sites and NPY-mediated effects, whereas the antagonism of M32 on exogenously applied galanin-induced reflex facilitation is postsynaptic on second order spinal neurones. Thus, involvement of a NPY receptor activation as part of the M32-mediated galanin receptor antagonism is unlikely.

Based on the differential pharmacological effects of these chimeric peptides, especially that of M40, the presence of galanin receptor subtypes has been suggested (Bartfai *et al.*, 1993b; Wynick *et al.*, 1993). Hypothalamic and hippocampal galanin receptors represent a putative central galanin receptor subtype (GL_1-receptor) which is blocked by M40 (Bartfai *et al.*, 1993b; Crawley *et al.*, 1993), whereas the pancreatic galanin receptor represents a peripheral subtype (GL_2-receptor) which recognizes M40, but as a weak agonist. The galanin receptors in the spinal cord (GL_1) may occupy an intermediate position between these two putative subtypes. However, it should be noted that the discrepancy between *in vitro* and *in vivo* results with these chimeric peptides may also be explained in terms of different metabolic stability, diffusion rates and penetration to the site of action. Recent cloning of galanin receptors from the human Bowes melanoma cell line (Habert-Ortoli *et al.*, 1994) opens the possibility of finally revealing in terms of their amino acid sequences the galanin receptor subtypes whose classification so far is based on pharmacological studies.

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A re-evaluation of the role of α_2-adrenoceptors in the anxiogenic effects of yohimbine, using the selective antagonist delequamine in the rat

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1 The acute behavioural effects of the α_2-adrenoceptor antagonists, yohimbine, idazoxan and delequamine (RS-15385-197) were compared in two tests of exploratory behaviour in the rat, operated in tandem. These were the elevated X-maze test (5 min) and a modified holeboard test (12 min), which comprised a holeboard arena with a small roof in one corner as a 'refuge'. Rats were first placed into this corner, thus enabling measurements of initial emergence latency and the number of forays. The experiments were always done with a concomitant vehicle control group, with 10–12 rats per group, and with the treatment blinded.

2 In order to validate the tests, the effects of representatives of four classes of psychoactive agents were examined, *viz.* picrotoxin (anxiogenic), chlordiazepoxide (anxiolytic), (+)-amphetamine (stimulant) and diphenhydramine (sedative). The modified holeboard tended to be more sensitive than the measurement of total arm entries in the elevated X-maze at detecting drug effects on exploratory behaviour, but unlike the X-maze it could not clearly identify each class of agent. Thus, picrotoxin (5 mg kg^{-1}, i.p.) reduced total arm entries and open arm exploration in the X-maze ($P<0.02$) and suppressed most measures of activity in the holeboard ($P<0.05$); chlordiazepoxide (7.5 mg kg^{-1}, i.p) increased total arm entries and open arm exploration ($P<0.02$) in the X-maze, without clear-cut effects in the holeboard; (+)-amphetamine (1 mg kg^{-1}, i.p.) had no significant effects in the X-maze, but increased most holeboard activities ($P<0.05$), and diphenhydramine (30 mg kg^{-1}, i.p.) reduced total arm entries in the X-maze ($P<0.002$) and hole exploration in the holeboard ($P<0.05$).

3 The actions of yohimbine most closely resembled those of picrotoxin. In the elevated X-maze, yohimbine (3 mg kg^{-1}, i.p.) decreased the total number of arm entries ($P<0.02$); a larger dose (10 mg kg^{-1}, i.p.) also reduced time spent on the open arms ($P<0.02$). In contrast, delequamine (3 mg kg^{-1}, i.p.) and idazoxan (3 mg kg^{-1}, i.p.) had no effect.

4 In the partially-shaded holeboard, yohimbine (3 mg kg^{-1}, i.p.) suppressed hole exploration ($P<0.05$); a higher dose (10 mg kg^{-1}, i.p.) increased emergence latency ($P<0.002$) and virtually abolished all activity. Delequamine (3 mg kg^{-1}, i.p.) and idazoxan (3 mg kg^{-1}, i.p.) did not influence emergence latency or holeboard activities.

5 The extent of the blockade of central α_2-adrenoceptors achieved during the tests was assessed by the ability of the doses used to reverse mydriasis induced by clonidine (300 μg kg^{-1}, s.c.) in anaesthetized rats. At a dose of 3 mg kg^{-1}, i.p., delequamine and idazoxan produced a rapid, sustained reversal of the clonidine response (by 87±2 and 86±2% respectively, 30 min after injection) whereas yohimbine produced a partial reversal of only 43±13%. The higher dose of yohimbine used in the exploratory tests (10 mg kg^{-1}, i.p.) was required in order to achieve 77±4% reversal of clonidine-induced mydriasis.

6 We therefore conclude that blockade of central α_2-adrenoceptors *per se* does not have an anxiogenic effect, at least in the rat. Thus, yohimbine is not an ideal tool for studying α_2-adrenoceptor function in animals and some of the anxiogenic effects of yohimbine previously ascribed to α_2-adrenoceptor antagonism may be secondary to other effects of this poorly selective compound.

Keywords: Delequamine; RS-15385-197; yohimbine; idazoxan; α_2-adrenoceptors; anxiety; anxiogenic; X-maze; plus-maze; holeboard

Introduction

Since the original report by Holmberg & Gershon (1961), several groups have confirmed that the indole alkaloid, yohimbine, can induce feelings of anxiousness, fear or panic in humans. In normal subjects the effect tends to be mild anxiety (Uhde *et al.*, 1984; Mattila *et al.*, 1988; Krystal *et al.*, 1992); in patients with agoraphobia or panic disorder the agent induces an enhanced anxiety response or panic (Charney *et al.*, 1987), and in patients with post-traumatic stress disorder, yohimbine can induce panic and flashbacks (Southwick *et al.*, 1993). In contrast, patients with generalized anxiety disorder (Charney *et al.*, 1989) or depression (Heninger *et al.*, 1988) show mild anxiogenic responses to yohimbine similar to those of normal subjects. Yohimbine has therefore proved useful in teasing out sub-groups of responders, but this is only of value from an aetiological point of view if its precise pharmacological mechanism of action is understood.

Following the discovery of presynaptic α_2-adrenoceptors (see Langer, 1974) located both terminally and somato-dendritically on central noradrenergic neurones, and the actions of yohimbine at these sites, increasing noradrenaline release at synapses (Dietl *et al.*, 1981) and the firing rate of neurones in the locus coeruleus (Rasmussen & Jacobs, 1986) respectively, it has largely been assumed that yohimbine's anxiogenic effect in humans was due to these actions. In support of this view,

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